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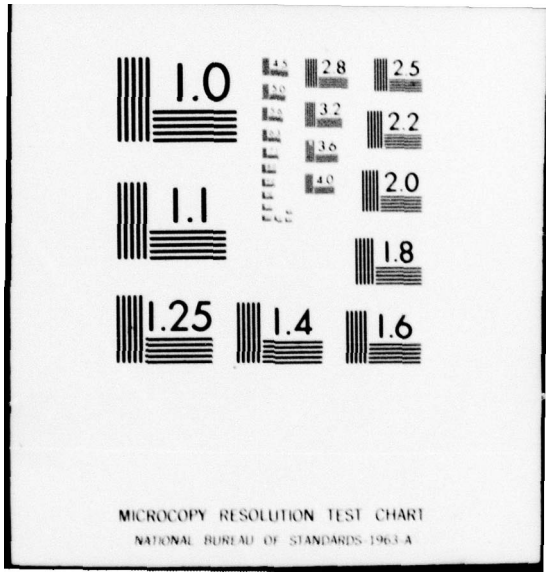
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The major accomplishment of the past year of research ~~supported by Contract No. DAMD 17-76-C-6076~~ was the demonstration that anergy in patients following major trauma and burns, as revealed in vivo by lack of delayed hypersensitivity responsive-

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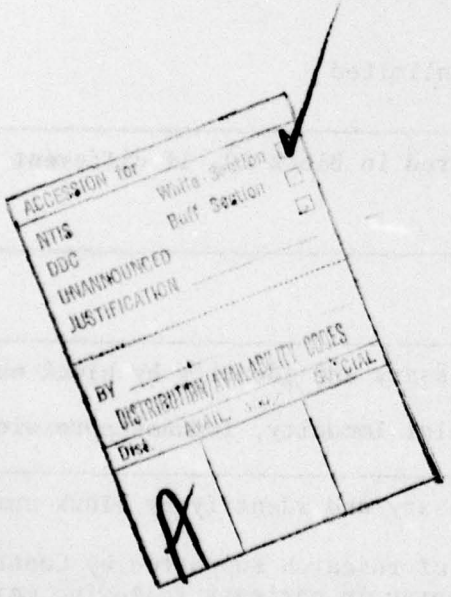
ness, was accompanied by and correlated with high levels of immunosuppressive activity in the patient's serum. We have also demonstrated that the ability of peripheral blood lymphocytes from trauma patients to form T-cell rosettes in vitro is associated with high levels of circulating immunosuppressive activity in the serum. This association is further borne out by the fact that patients whose lymphocytes form diminished numbers of T-cell rosettes, as compared with control individuals frequently show markedly improved rosette forming ability after multiple in vitro washings of their lymphocytes. We have begun to characterize the suppressive material washed from the lymphocyte's surface and have defined an active fraction by gel exclusion chromatography. We have also determined that while the peripheral blood lymphocytes of patients following trauma and burns frequently demonstrate diminished responsiveness to stimulation by phytohemagglutinin (PHA) in tissue culture, diminished PHA responsiveness is not correlated with the presence or absense of circulating immunosuppressive polypeptide material in the serum.



PROGRESS REPORT - ANNUAL

B. Annual Progress Report - During the past year we have looked for reduced capacity for activation of peripheral blood lymphocytes from 24 patients who had suffered operative or accidental trauma or had sustained major burns. The peripheral blood lymphocytes of these patients were tested for their ability to respond to PHA stimulation in vitro at three PHA concentrations and were compared with simultaneously tested peripheral blood lymphocytes from normal individuals. Tests were performed before and after multiple washings in tissue culture medium to determine whether or not washing of the lymphocytes would increase their responsiveness to mitogens as we had noted when studying the peripheral blood lymphocytes of patients with metastatic cancer (Cancer Res. 37:3022-2025, 1977). The results of this work are summarized in Tables I and II. It is apparent that there is no consistent pattern seen with regard to lymphocyte activation in patients after major operative trauma or burns. The results of testing of lymphocyte activation also do not appear to correlate with the presence or absence of immunosuppressive serum, as defined by the ability of patients' serum in 10% concentration to suppress by 50% or more the response of peripheral blood lymphocytes from normal human volunteers to PHA stimulation in vitro. It is apparent, however, that as we had found in studying cancer patients, the lymphocytes of trauma patients were significantly more likely to increase (defined in these experiments as a doubling of ^{3}H-thymidine incorporation) their maximal response to PHA after six washings in vitro if their response to PHA had initially been diminished (defined as 50% or less of simultaneous controls). The latter finding suggests that in at least some trauma patients diminished lymphocyte activation may be associated with loosely bound suppressive material on the cell membrane. We have also studied the E-rosette-forming (to sheep red blood cells) ability of peripheral blood lymphocytes from traumatized and burn patients. At present there is a suggestive, though not yet statistically significant, correlation between the presence of suppressive serum and the ability of patients' peripheral blood lymphocytes to form E-rosetteswhen resuspended in normal tissue culture medium when compared with lymphocytes from normal controls (Table III). Study of more patients is obviously necessary to clarify this issue. It seems clear, however, that six washings in vitro leads to a significant (defined as 50% or more) increase in E-rosette formation by lymphocytes from more than half the patients whose lymphocytes showed diminished rosette formation without multiple washings. The same response to multiple washings was not found with lymphocytes from patients or controls who initially formed normal percentages of E-rosettes. (Table IV)

We have attempted to characterize the material washed off the lymphocytes from those trauma patients whose lymphocytes showed an increased response to PHA or an increased ability to form rosettes after multiple washings in tissue culture medium. We placed the medium in which the lymphocytes had been washed on an Amicon UM-05 ultrafilter in order to remove salt and low molecular weight substances. The remaining higher molecular

weight material was then recovered by lyophilization, redissolved in phosphate buffered saline and chromatographed on G25 Sephadex. Suppressive activity was consistently recovered from this material in the fourth peak off the Sephadex column. The active material was ninhydrin positive and presumably contained polypeptide; however, it was very heterogenous by high voltage electrophoresis. Material recovered from the medium in which normal lymphocytes had been washed also contained suppressive material which was recovered in the same peak after-G25 Sephadex chromatography. We have thus not been able to demonstrate that a unique molecular species with suppressive activity has been recovered by washing lymphocytes from traumatized patients nor have we yet been able to demonstrate that a greater quantity of suppressive material is recovered by washing patients' lymphocytes than is recovered by washing the lymphocytes of normal individuals. Obviously this question needs to be settled by further studies.

During the past year we have been pooling the serum obtained from traumatized patients who have shown significant suppressive activity in their serum defined, as noted above, by the ability of the serum in 10% concentration to significantly suppress without toxicity the PHA stimulation of peripheral blood lymphocytes from normal human volunteers. This pooled serum has recently been fractionated by DEAE cellulose chromatography and the suppressive activity has been largely recovered in Peak I eluted from the DEAE column with 0.005 M acetate buffer. This confirms our findings previously published (Ann. Surg. 185:73, 1977). We now plan to obtain an active peptide moiety from this Peak I material by acidification with acetic acid to pH_3 and diafiltration on Amicon UM-2 membranes. The recovered low molecular weight peptide fraction will be utilized to complete the experiments described below to determine its effect on the ability of mice to resist Listeria infection.

In searching for sufficient quantities of starting material for final purification of the immunosuppressive peptide fraction in the plasma and body fluids of patients who have suffered trauma or burns we have recently found that the urine of such individuals contains considerable quantities of immunosuppressive peptide. We have recently begun to process urine from trauma patients by removing salt and low molecular weight substances by washing on a UM-05 membrane followed by lyophilization and partitioning on a UM-2 ultrafilter. The low molecular weight fraction passing through the ultrafilter is then chromatographed on G25 Sephadex. Finally, high voltage electrophoresis will be used as a purification step as outlined below.

During the latter part of the past year we have begun to study the delayed hypersensitivity responsiveness of a series of patients before and after operative trauma of various sorts. We have skin tested these patients for common recall antigens before and after surgery. The antigens used

were mumps, SK/SD, and PPD. We have also attempted to sensitize certain of these patients to DNCB. We have also drawn serum from all of these individuals for measurement of serum suppressive activity. As patients who have undergone minor trauma, we have utilized 10 individuals having inguinal herniorrhaphy under general anesthesia. As examples of patients undergoing major trauma we have tested 19 patients who have had either abdominal aortic aneurysm resections or have undergone coronary artery bypass grafting. These individuals have been skin tested pre-operatively, at 48-72 hours following surgery, 5-7 days following surgery and in some cases 10-12 days following surgery. We have also skin tested each of them in the late post-operative period, i.e., two weeks or more after surgery. The results are summarized in Table V and Figure 1. The table divides patients into groups depending upon their response to skin test antigens 48-72 hours following surgery. Anergy was considered a lack of response to all of the recall antigens and failure to be sensitized to DNCB. A patient was considered responsive if he manifested a clear-cut delayed-type reaction to one or more of the antigens. Suppressive serum in these individuals was defined as noted above. Patients' serum was always compared with pooled normal serum used in the same concentration. 5% fetal calf serum was always added to each lymphocyte culture in order to provide sufficient serum factors to permit maximal PHA stimulation.

It is apparent from Table V that none of the patients who underwent inguinal herniorrhaphy were anergic 72 hours post-operatively and that none of them had suppressive serum. Of the 19 patients undergoing major surgical trauma, 8 were anergic 72 hours post-operatively and 7 of the 8 had suppressive serum. 11 were not anergic and 2 of the 11 had suppressive serum. The correlation of anergy with suppressive serum is statistically significant. The anergic and non-anergic patients in the major trauma group did not differ with respect to the number of blood transfusions received and none were given antibiotics known to suppress lymphocyte activation. As noted in Figure 1, the time course of anergy and suppressive activity of serum also appeared to be the same following major surgery. The patients who were anergic on the second or third post-operative day remained anergic for the first post-operative week. The suppressive activity of the serum in the anergic and in the responsive individuals, respectively, is also shown in Figure 1. It is apparent that the anergic patients as a group developed significantly suppressive sera which returned to a normal non-suppressive state in the late (beyond 14 days) post-operative period at which time the patients had recovered skin test reactivity as well. The difference between the anergic and reactive patients with respect to suppressive serum remains highly significant throughout the post-operative course.

We believe these preliminary results strongly suggest that there is, in fact, a correlation between deficient cellular immunity as manifested *in vivo* and factors (s) in the serum suppressive of lymphocyte activation *in vitro* in a group of patients subjected to operative trauma and that the manifestations of deficient cellular immune reactivity are roughly correlated with the severity of the trauma.

TABLE I

PHA stimulation of peripheral blood lymphocytes from 24 trauma patients studied on 60 occasions.

Patients' Serum	Diminished PHA Response	Normal Response
Suppressive - 30	10	20
Non-suppressive - 30	7	23

$$Chi^2 = 0.3283$$

$$p > 0.5$$

TABLE II

Effect of washing In Vitro on PHA Response of Lymphocytes from Trauma Patients

Initial Response	Response After 6 Washes In Vitro: Increased	Not Increased
Diminished - 17	10	7
Normal - 43	5	38
Controls - 60	3	57

Ch^2 Diminished vs Normal = 12.0657, $p < 0.001$

Chi^2 Diminished vs Controls = 23.6461, $p < 0.00001$

Ch^2 Normal vs Controls = 0.7501, $p > 0.3$

TABLE III

E-Rosette Formation by Lymphocytes from 24 Trauma Patients Studied on 45 Occasions.

Patients' Serum	Rosettes Diminished	Rosettes Normal
Suppressive - 24	16	8
Non-Suppressive - 21	8	13

Chi^2 2.61

$p \approx 0.1$

TABLE IV

Effect of Washing In Vitro on E-Rosette Formation by Lymphocytes of Trauma Patients.

Initial Rosettes	Rosettes After 6 Washes In Vitro: Increased	Not Increased
Diminished - 24	14	10
Normal - 21	0	21
Controls - 45	0	45

Chi^2 Diminished vs Normal = 15.1646, $p < 0.00001$

Chi^2 Diminished vs Controls = 29.4238, $p < 0.00001$

TABLE V

CORRELATION OF ANERGY WITH SUPPRESSIVE SERUM

AFTER SURGICAL TRAUMA

SURGERY	SKIN TEST	IMMUNOSUPPRESSIVE SERUM *	NON-SUPPRESSIVE SERUM	TOTAL NO.
MINOR	Positive	0	10	10
	Negative	0	0	0
MAJOR	Positive	2	9	11
	Negative	7**	1	8

* Immunosuppressive serum at 10% concentration was more than 50% suppressive of PHA stimulation of normal human lymphocytes.

** CHI Square with Yate's Correction: $*p < 0.05$.

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FIGURE 1